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Combined nitrification-denitrification processes

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Abstract: Nitrification and denitrification have traditionally been regarded as essentially separate phenomena, carried out by different bacteria in segregated areas of soil, sediments, water or reactors. However, research in the 1980s and 1990s has established that nitrifiers and denitrifiers are not as metabolically fastidious as previously thought, and strict segregation is not necessary. Moreover, some bacteria are able to convert NH_4^+ and other reduced nitrogen compounds to nitrogen gas and the gaseous nitrogen oxides in combined nitrification/denitrification processes. Such organisms are of interest for wastewater treatment for two opposing reasons. Firstly, the idea of single-stage nitrogen removal has obvious attractions for system design. Secondly, N_2O is a serious pollutant, implicated in virtually all current environmental problems (e.g. acid rain, greenhouse effect, ozone depletion).

Key words: Nitrification; Denitrification; N_2O; N_2; Wastewater

Introduction

Nitrification and denitrification processes are traditionally regarded as separate, occurring in different layers of water, soils and sediments, and requiring individual reactors for separate waste treatment. However, current research in different countries has revealed that the situation is not so clear-cut. Nitrification and denitrification can take place simultaneously in microbial communities, co-cultures and even in pure cultures. With our increasing awareness of the need to control the emission of gaseous and dissolved nitrogen compounds, the possibility of single-stage nitrogen removal has obvious attractions, and understanding of the combined processes is needed in order to be able to encourage the activity of bacteria capable of carrying it out. Indeed, as Abeliovich [1] points out, even the control of nitrification (and denitrification) must be a laborious procedure of trial and error, unless the processes are fully understood. There are more negative reasons why combined nitrification–denitrification processes require understanding. For example, if, as seems likely, nitrifiers and denitrifiers are contributing significantly to the production of NO_x gases (see below), the mechanisms underlying such emissions must be understood in order to be able to prevent them. This paper will review combined nitrification–denitrification processes in different types of bacteria, and will consider the parameters that are critical to ensure that the only environmentally safe nitrogen product, N_2, is the sole product.

The autotrophic nitrifiers

Until a few years ago, the metabolism of the autotrophic nitrifiers was believed to be relatively

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Table 1

Production of ^{15}-labelled gases from $^{15}NO_2^-$ (20 μmol) and nitrite production from added ammonium (375 μmol) by two known nitrifiers and a newly isolated nitrifying species in statically incubated batch cultures with air in the headspace [3]

Organism	N_2O ($\mu l\,l^{-1}$)	$^{14,15}N_2$ (nmol)	$^{15,15}N_2$ (nmol)	NO_2^- (μmol)
None	< 1.0	−2.2	1.4	20
Nitrosomonas europaea	216	1.0	2.3	392
Nitrosolobus multiformis	< 1.0	2.4	3.7	394
Nitrosomonas sp.	< 1.0	81.4	87.4	394

simple. Obligately aerobic ammonia oxidizers made nitrite, and obligately aerobic nitrite oxidizers made nitrate. This situation was considered to be so stable that the two end products, nitrite and nitrate, were used as measures of nitrification efficiency. The first indication that things were not that simple came with the work of Poth [2,3], who showed that the ammonia oxidizers could produce NO, N_2O and even N_2 under suitable experimental conditions. Table 1 shows the production of these gases by different ammonium-oxidizing autotrophs in cultures in which oxygen was limiting because the flasks were statically incubated. All three strains reacted differently. The 'classical' nitrifier, *Nitrosomonas europaea* produced a significant amount of N_2O, *Nitrosolobus multiformis* produced very little gas, but an unidentified *Nitrosomonas* strain produced N_2. These observations indicate that the nitrite-producing autotrophs have mechanisms for coping with oxygen-limitation, or even anaerobiosis. Abeliovich [4] showed that both *Nitrosomonas* and *Nitrobacter* species are common in the anaerobic areas of wastewater reservoirs. Indeed, it has since been shown that *N. europaea* was able to use nitrite as its electron acceptor under strictly anaerobic conditions if pyruvate was provided as an energy source [5]. However, Remde and Conrad [6] showed that both *N. europaea* and *Nitrosovibrio* strain K71 generated NO, even under aerobic conditions (approximately 1.7 and 0.75 nmol min^{-1} (mg biomass)$^{-1}$, respectively). N_2O was only produced in significant amounts after the culture was suddenly switched to anaerobiosis (approximately 45.6 and 4.7 nmol min^{-1}

Table 2

Examples of prokaryotic and eukaryotic heterotrophs which nitrify (data mainly from [27])

Species	Substrate	Product
Arthrobacter globiformis	Ammonium	Hydroxylamine
Aspergillus flavus	Ammonium	Monohydroxamic acid
Streptomyces sp.	Ammonium	Monohydroxamic acid
Mycobacterium phlei	Ammonium	Dihydroxamic acid
Aerobacter aerogenes	Ammonium	Dihydroxamic acid
Rhodotorula sp.	Ammonium	Dihydroxamic acid
Ustilago sphaerogena	Ammonium	Trihydroxamic acid
Neurospora crassa	Ammonium	Trihydroxamic acid
Streptomyces griseus	Ammonium	Trihydroxamic acid
Thiosphaera pantotropha	Ammonium	Nitrite *
Proteus sp.	Hydroxylamine	Nitrite
Alcaligenes sp.	Oximes	Nitrite *
Pseudomonas aeruginosa	Aliphatic nitro compounds	Nitrite
Flavobacterium sp.	Aromatic nitro compounds	Nitrite
Nocardia sp.	Aromatic nitro compounds	Nitrite
Chlorella sp.	Ammonium	Nitrate
Aspergillus parasiticus	Ammonium	Nitrate
Aspergillus wentii	Nitrite	Nitrate
Aspergillus flavus	Aliphatic nitro compounds	Nitrate
Pseudomonas sp.	Aromatic nitro compounds	Nitrate

* Bacteria known to simultaneously denitrify to N_2O or N_2.

$$NH_4^+ \longrightarrow NH_2OH \longrightarrow NO_2^- \longrightarrow NO_3^-$$

The inorganic pathway of nitrification

$$RNH_2 \longrightarrow RNHOH \longrightarrow R\text{-}NO \longrightarrow RNO_2 \longrightarrow NO_3^-$$

The organic pathway of nitrification

Fig. 1. The two pathways of nitrification potentially available to heterotrophic nitrifiers. Of course, combinations of the two are possible [31].

(mg protein)$^{-1}$, respectively), when NO production also accelerated (to approximately 5.4 and 1.8 nmol min^{-1} (mg biomass)$^{-1}$, respectively). A rough calculation, based on nitrification rates obtained with continuous cultures of *N. europaea* [7] indicates that aerobic NO production by these bacteria represents around 0.1% of the total nitrification. Short-term exposure of *N. europaea* to anaerobiosis increased this to 0.5%, with 3.9% of the output from nitrification going to N_2O. This is not only important for wastewater treatment systems, where almost any reactor involving biofilms will have anoxic areas, but also for natural conditions and certainly in fertilizer management where all but the driest of soils will have anaerobic microsites [8].

As well as the ammonia oxidizing bacteria, some of the nitrite oxidizers also appear to posses unexpected properties that have changed the old picture of nitrification. For example, it has been shown that some nitrite oxidizers are not obligate autotrophs, but mixotrophs [9], and some *Nitrobacter* species can even denitrify if grown as heterotrophs [10].

The heterotrophic nitrifiers

Heterotrophic nitrifiers oxidize a range of reduced nitrogen compounds, apparently without gaining energy from the reaction. Indeed, an organic source of energy is generally necessary. The phenomenon has been known since the time of Winogradsky, and probably covers a group of physiologically and biochemically diverse, although superficially similar, reactions. It has been observed in bacteria, fungi, algae and cells from more complicated tissues (e.g. rat liver), as well as in bacteria (Table 2). There are certainly at least two distinct pathways involved (Fig. 1). Moreover, the scope of heterotrophic nitrifiers is wide, cov-

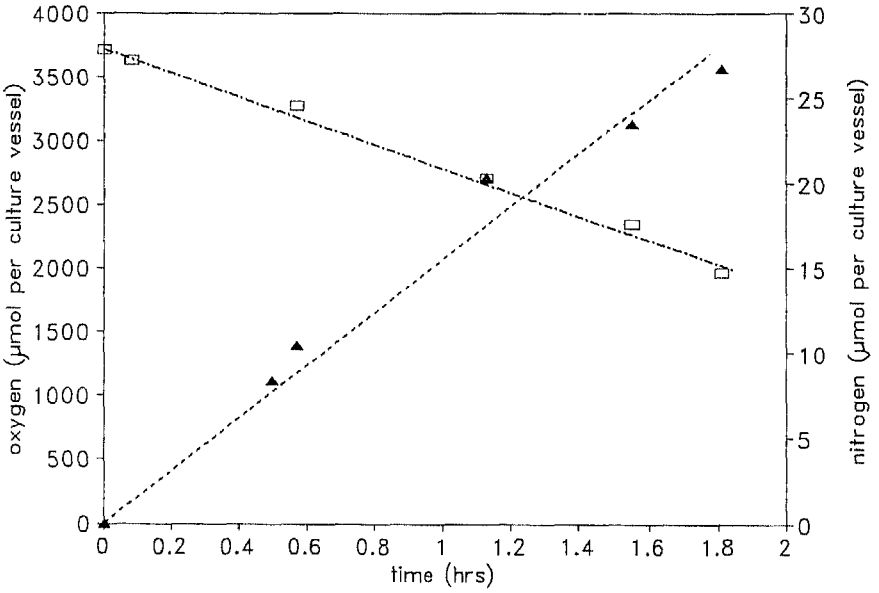

Fig. 2. Oxygen disappearance (□) and nitrogen appearance (▲) in the headspace of an aerobic batch culture of *Thiosphaera pantotropha* growing on acetate (L.A. Robertson and T. Dalsgaard, unpublished data).

ering compounds as disparate as ammonium and nitro-aromatics (Table 2), and probably serving various different functions. For example, Verstraete and Alexander [11] showed that heterotrophic nitrification in *Arthrobacter* sp. forms part of the response of the organism to iron limitation. If iron concentrations are limiting, chelating hydroxamates are synthesized. If iron is in excess, nitrite and nitrate are generated instead. In *Thiosphaera pantotropha* and a few other species, it seems that heterotrophic nitrification serves as a means of dumping excess reducing power [12].

The heterotrophic nitrifiers merit inclusion in this paper because most of them are also denitrifiers [13]. A number of them have been found to be able to denitrify aerobically (for reviews see [14,15]), and to be able to reduce nitrification products (nitrite and nitrate) as they are generated. Studies with *T. pantotropha* initially suggested that the situation was simple. Nitrite was produced from ammonium, and then denitrified (together with exogenously supplied nitrite or nitrate) by the denitrification pathway. However, with extended culturing, *T. pantotropha* has gradually lost its aerobic denitrifying capacity. This has had several consequences for the behaviour of the species. For example, the μ_{max} has increased substantially, and the organism no longer produces the mesosome-like membrane structures observed with the original strain [16]. However, *T. pantotropha* continues to nitrify, and nitrite does not accumulate in the cultures. Indeed, experiments using gas chromatography and mass spectrometry have recently confirmed that N_2 is produced from NH_4^+ by *T. pantotropha* in well-mixed, aerobic batch cultures sparged with a He/O_2 mixture. Fig. 2 shows simultaneous oxygen uptake and N_2 production by one such culture (Robertson and Dalsgaard, unpublished data). If $^{15}NO_2^-$ was supplied to the culture, the label did appear in the gas produced, but nitrogen production rates were not significantly different from when only NH_4^+ was supplied (4.9 and 5.3 nmol min^{-1} mg $protein^{-1}$, respectively), suggesting that the organism retained only a limited nitrite reduction capacity, sufficient to cope with the amount of nitrite generated by nitrification. Other species continue to denitrify aerobically, but may show slightly different behaviour if presented with NH_4^+ alone, or with NH_4^+ together with NO_3^- or NO_2^-. Thus aerobic (> 90% air saturation) batch cultures of *Alcaligenes faecalis* TUD produced both N_2 (29.1 nmol min^{-1} mg $protein^{-1}$) and N_2O (12.2 nmol min^{-1} mg $protein^{-1}$) when provided with NH_4^+ and NO_2^-. Subsequent experiments with cultures in aerobic (dissolved oxygen 50% air saturation, stirrer speed > 800 rpm) batch cultures produced $^{15,15}N_2$ from $^{15}NH_4^+$, but equal amounts of $^{14,14}N_2O$, $^{14,15}N_2O$ and $^{15,15}N_2O$ from a mixture of $^{14}NH_4^+$ and $^{15}NO_2^-$ (Robertson and Kuenen, unpublished data). It should be noted that heterotrophic nitrification/denitrification rates increase as the dissolved oxygen falls.

Co-cultures and mixed cultures of nitrifiers

Once it was realised that (heterotrophic) nitrification rates could not be accurately estimated from nitrite (or nitrate) concentrations, it became clear that some heterotrophic nitrification rates were actually much higher than previously realised. Given the larger numbers of heterotrophs present in both natural and man-made situations, heterotrophs might make a significant contribution to the total nitrification under some conditions. Indeed, prior to this, Castignetti and Gunner [17] had shown that an ammonium-oxidizing heterotroph was capable of producing sufficient NO_2^- to support growth of a *Nitrobacter* species (Fig. 3). Measurements based on nitrogen balances revealed that, while still lower per unit of biomass than autotrophic nitrification rates, heterotrophic nitrification rates were at least an order of magnitude higher than previously believed (Table 3). Hence, if the biomass made up of heterotrophic nitrifiers was 20–50 times higher than that of autotrophic nitrifiers, the total conversion rates of the heterotrophic nitrifiers could easily match those of the autotrophs. This would not only be important for our understanding of the control mechanisms underlying nitrogen-cycling in natural habitats (e.g. sediments, water bodies), but certainly also for wastewater treat-

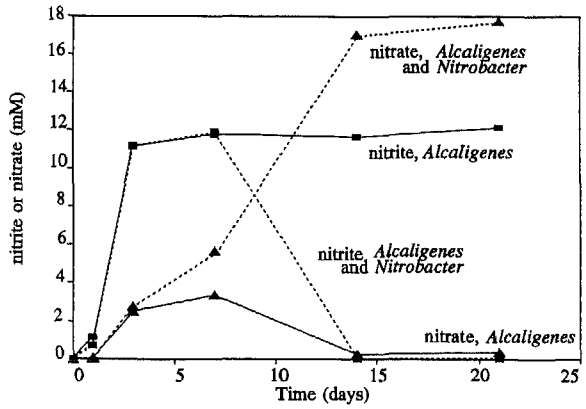

Fig. 3. Nitrate (▲) and nitrite (■) levels in axenic cultures of *Alcaligenes* sp. (solid lines) and mixed cultures of *Alcaligenes* and *Nitrobacter* species (broken lines), growing on pyruvic oxime [17].

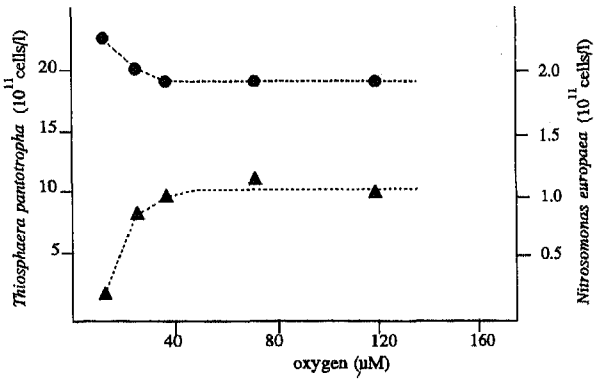

Fig. 4. Response of mixed continuous cultures of *Thiosphaera pantotropha* (●) and *Nitrosomonas europaea* (▲) to different levels of dissolved oxygen [32].

ment. One might even ask whether heterotrophic nitrifier/denitrifiers might present a viable option for nitrogen removal from some wastewaters, especially since they grow more rapidly than the autotrophs. A possible advantage of such a system might be that the bacteria would remove any organic materials in the wastewater, and that at least some of the NO_2^- produced by heterotrophic nitrification would be immediately converted to N_2. In order to obtain quantitative data on the relative importance of autotrophic nitrification and the heterotrophic combination of nitrification and denitrification, competition experiments between a representative heterotrophic nitrifier (*T. pantotropha*) and a representative autotrophic ammonium oxidizer (*N. europaea*) were therefore set up in order to examine the influence of two potentially critical parameters, O_2 (Fig. 4) and the C:N ratio (Fig. 5). It can be seen that, as might be expected, the het-

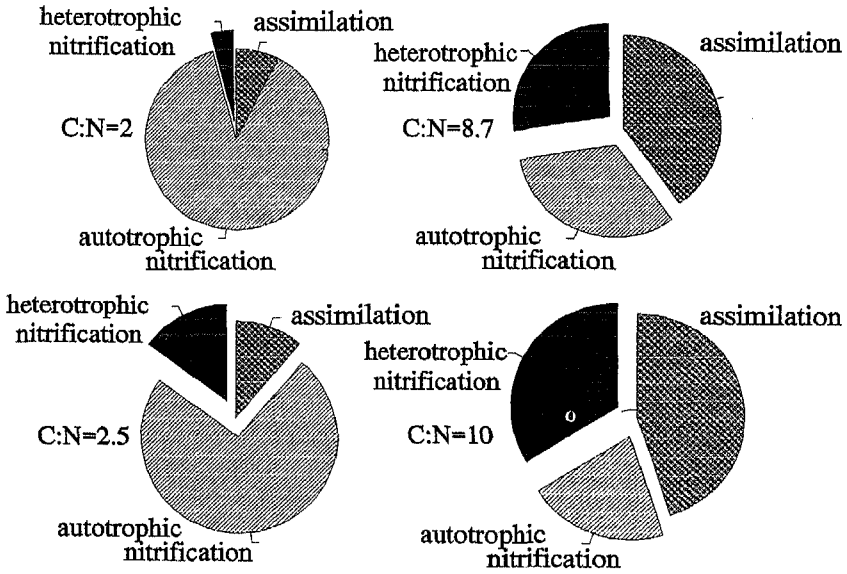

Fig. 5. Fate of NH_4^+ in co-cultures of *Thiosphaera pantotropha* and *Nitrosomonas europaea* at different C:N ratios (data from [32]).

erotrophic nitrifier did best at low dissolved oxygen concentrations, although it was possible to run relatively stable co-cultures of the two species over much of the O_2 and C:N ranges. The large proportion of nitrogen being assimilated (Fig. 5) at the higher C:N ratios should be noted, as this emphasizes the higher sludge production that would be associated with nitrification reactors utilizing heterotrophs rather than autotrophs. At the high C:N ratios, the heterotrophic nitrification/denitrification rates (measured as nitrogen losses) were, again, in the order of 5–20 nmol min^{-1} mg $protein^{-1}$, confirming the rates mentioned above. Subsequent experiments using a fill-and-draw reactor rather than a chemostat gave essentially similar results (M. Pot, unpublished results).

Heterotrophic nitrification, being strictly dependent on the presence of a suitable organic carbon and energy source, falls into the general metabolic category of co-metabolic conversions, whereby the product (in this case NO_2^-) can be used by the same or another organism for further metabolism. The examples of two-membered cultures described above clearly demonstrate this principle. Another interesting example of co-metabolic (heterotrophic) nitrification was revealed by analysis of a community growing on a CH_4/NH_4^+ medium [18,19]. The culture was found to be producing N_2O (Fig. 6). It appeared that during CH_4 oxidation, the methanotroph was also co-oxidizing NH_4^+ to NH_2OH. This potentially inhibitory compound was then being nitrified by pseudomonads growing on metabolites

Table 3

Nitrification rates (nmol NH_3 min^{-1} (mg dry weight)$^{-1}$) calculated from published batch culture results

Organism	Activity	N-compound used
Pseudomonas aeruginosa	12–28	Hydroxamate [28]
Pseudomonas aeruginosa	70–90	Hydroxylamine [28]
Alcaligenes sp.	33	Pyruvic oxime [29]
Pseudomonas denitrificans	2.6	Pyruvic oxime [13]
Thiosphaera pantotropha	35.4	Ammonia [7]
Nitrosomonas sp.	130–1550	Ammonia [30]

If other nitrogen compounds were originally used, the results have been re-calculated as though for ammonia (references in square brackets).

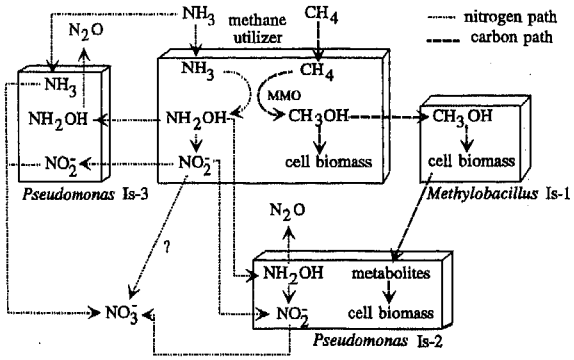

Fig. 6. Interactions between the four members of a mixed culture growing on CH_4 and NH_4^+ (data from [19]).

excreted by the methanotroph and methylotroph, and producing N_2O. NO_3^- was the main nitrification product of the co-culture, but as with the N_2O generation, its production from NO_2^- was dependent on CH_4 oxidation. When the culture stopped growing because of CH_4 depletion, NO_2^- oxidation also ceased. This example demonstrates the complexity of nitrogen cycling, even in relatively simple microbial systems or habitats, and explains the need for further quantitative understanding of the microbial interactions involved in nitrification and denitrification, especially where different end products are involved.

The conversion of NH_4^+ to gaseous products has also been shown [20] to occur in sludge from wastewater treatment plants that had a record of 'losing' nitrogen. Sludge sample were taken, homogenized well to avoid clumps, and then vigorously aerated, and supplied with $^{15}NH_4^+$. Analysis of the gas stream revealed that the primary nitrogenous gas was N_2O, rather than N_2.

In the context of nitrogenous gas production from NH_4^+, Bremner and Blackmer [21] showed that NO and N_2O emissions from maize fields were highest when $(NH_4)_2SO_4$, urea or alanine were used as fertilizer (Table 4). It was unlikely that denitrifiers were the direct cause, as gas production was much lower when NO_3^- was used. However, it is clear from these data that a considerable amount of nitrogenous fertilizer is wasted. It may be expected that, with understanding of the factors governing nitrification–denitrification in the field, the amount of fertilizer required by

Table 4

Effect of various additives on N_2O emission (as ng/g soil) from well-aerated soil samples after 7 days incubation at 30°C

Treatment	Emission of N_2O (in ng N/g soil/7 days)	
	Harps soil	Webster soil
None	4	6
Ammonium sulfate	246	50
Urea	292	75
Alanine	218	81
Potassium nitrate	4	7
Glucose	1	5
Nitrate + glucose	4	8

All nitrogen compounds were supplied at a concentration giving 100 μg N/g soil. Glucose, when added, was at a concentration of 0.25 mg/g soil. The Harps soil contained 7.1% organic material and had a pH of 7.9. The organic content of the Webster soil was 10.2%, with a pH of 6.2. (data from [21]).

farmers could be reduced (and undesirable emissions avoided) by an informed selection of fertilizer type.

Anoxic ammonium oxidation

Thus far, only aerobic systems have been considered. In 1977, Broda described a number of "lithotrophs missing in nature", bacterial types that should exist on energetic grounds, but had never been isolated [22]. Among these were bacteria able to oxidize NH_4^+ to N_2 with NO_3^- or NO_2^- as the electron acceptor. The free energy

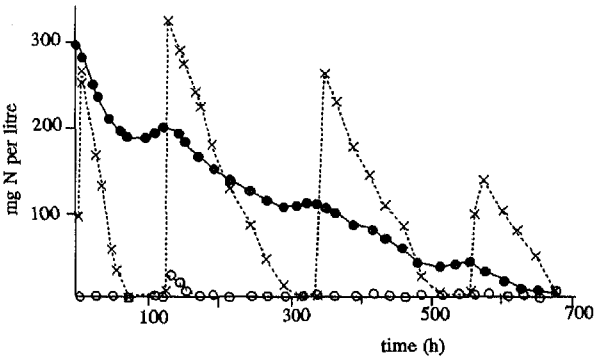

Fig. 7. The effect of nitrate pulses (×) on the NH_4^+ (●), NO_2^- (○) in anoxic batch cultures of 'anammox' sludge (data from [23]).

balance for this reaction is as favourable as when O_2 accepts the electrons:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O$$

$$\Delta G' = -361\ \text{kJ/mol}\ NH_4^+$$

$$NH_4^+ + 0.75\,O_2 \rightarrow 0.5\,N_2 + 1.5\,H_2O + H^+$$

$$\Delta G' = -315\ \text{kJ/mol}\ NH_4^+$$

Recently, it was noted that NH_4^+ was disappearing from a denitrifying reactor treating effluent from methanogenesis. The electron donors in this reactor were volatile fatty acids left over from methanogenesis and sulfide generated by the sulfate-reducing bacteria in the acetogenic and methanogenic reactors. Initially, the observed nitrate consumption agreed well with the theoretical nitrate requirement based on the concentrations of volatile fatty acids and sulfide (line I, Table 5). However, once NH_4^+ started to disappear, nitrate consumption increased, and the observed and calculated nitrate requirements did

Table 5

Nitrogen balances for the anammox reactor

	Amount of NO_3^- required for		Total NO_3^- required for		Measured NO_3^-	
	SO_4^{2-} formed	volatile fatty acids use	NH_3^- anammox	Without anammox	With anammox	Consumption
(I)	92	18	0	110	110	100
(II)	80	18	48	98	146	150

Data from [23].
(I) Before anammox; (II) after anammox appeared (all concentrations as mg N per litre).

not match until the amount stoichiometrically needed to oxidize the disappearing NH_4^+ was included in the calculation (line II, Table 5). Subsequent experiments [23,24] showed a clear correlation between NO_3^- and NH_4^+ disappearance (Fig. 7). Control experiments (e.g. using different amounts of live and dead biomass) confirmed that the reaction is indeed biological, and a $^{15}NH_4^+$ pulse into the reactor confirmed that the product of the reaction was mostly N_2. Rates of N-removal (total NH_4^+ and NO_x^-) compared very favourably with nitrification rates obtained in conventional systems of similar working volumes.

Conclusion

Nitrogen-associated pollution is increasing, with dire consequences for the environment. Eutrophication, acid rain, the greenhouse effect, all of these and more have contributions from nitrogen compounds in one form or another. Nitrifiers are reported to be to blame for much of the corrosion of sandstone masonry now observed, particularly among ancient monuments [25]. N_2O, for example, has an estimated lifetime of 130 years. Although its effect is not as spectacular as that of CO_2, it was predicted in the 1970s that a doubling of the N_2O concentration in the air would be sufficient to give the 1°C increase in average temperature necessary to make a major impact on world climate. Recent estimates suggest that this has already increased by 24% [26]. It is essential that we not only set up nitrogen-removing waste-treatment systems, but that we also understand the (eco)physiology of the bacteria involved. For example, the discussion above has shown that the processes of nitrification and/or denitrification are much more complex than previously thought. It is also clear that the contribution of the combined aerobic nitrification/denitrification and anoxic nitrification/denitrification to total nitrogen cycling, although occurring at low specific rates, may be substantial. Only with such additional insight can reactors be improved, and their limitations appreciated. Recent reports have suggested that 18–25% of the NO_x gas reaching the atmosphere originates from sub-optimal waste treatment reactors. Even apparently insignificant amounts on the laboratory scale can become considerable when scaled up to cover full-scale reactors. For example, an average wastewater treatment plant treating mixed domestic and industrial effluent can be estimated to treat 150 l of wastewater per person per day. This wastewater generally has a N-content between 40–60 mg per litre. Taking the lower value, this is equivalent to 2.2 kg N per person per year. Even if only 0.1% of this remains in the form of N_2O, emission will be considerable. New and novel systems must be understood, and optimized on the basis of this understanding, or we may simply be transferring the problem rather than solving it.

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